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Effects of Lactobacilli on Yeast-Catalyzed Ethanol Fermentations

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Normal-gravity (22 to 24° Plato) wheat mashes were inoculated with five industrially important strains of lactobacilli at ~10^5, ~10^6, ~10^7, ~10^8, and ~10^9 CFU/ml in order to study the effects of the lactobacilli on yeast growth and ethanol productivity. *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus* #3, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* were used. Controls with yeast cells but no bacterial inoculation and additional treatments with bacteria alone inoculated at ~10^7 CFU/ml of mash were included. Decreased ethanol yields were due to the diversion of carbohydrates for bacterial growth and the production of lactic acid. As higher numbers of the bacteria were produced (depending on the strain), 1 to 1.5% (wt/vol) lactic acid resulted in the case of homofermentative organisms. *L. fermentum*, a heterofermentative organism, produced only 0.5% (wt/vol) lactic acid. When *L. plantarum*, *L. rhamnosus*, and *L. fermentum* were inoculated at ~10^6 CFU/ml, an approximately 2% decrease in the final ethanol concentration was observed. Smaller initial numbers (only 10^5 CFU/ml) of *L. paracasei* or *Lactobacillus* #3 were sufficient to cause more than 2% decreases in the final ethanol concentrations measured compared to the control. Such effects after an inoculation of only 10^5 CFU/ml may have been due to the higher tolerance to ethanol of the latter two bacteria, to the more rapid adaptation (shorter lag phase) of these two industrial organisms to fermentation conditions, and/or to their more rapid growth and metabolism. When up to 10^9 CFU of bacteria/ml was present in mash, approximately 3.8 to 7.6% reductions in ethanol concentration occurred depending on the strain. Production of lactic acid and a suspected competition with yeast cells for essential growth factors in the fermenting medium were the major reasons for reductions in yeast growth and final ethanol yield when lactic acid bacteria were present.

Bacterial contamination is a major cause of reduction in ethanol yield during fermentation of starch-based feedstocks by *Saccharomyces cerevisiae*. Among the bacterial contaminants encountered, the lactic acid bacteria are the most troublesome because of their tolerance to high temperature and low pH and their ability to grow rapidly. The genus *Lactobacillus* is of major concern to distilleries and fuel alcohol plants, whereas in breweries, the genus *Pediococcus* is a persistent contaminant which often produces off-flavors. As flavor is very important in beer, management practices in breweries have been established to keep these bacteria to a minimum. In distilleries, cleaning and sanitizing are much less rigorous, and mashes are subjected to less heat and are not sterile. Contaminants can arise from tankage, transfer lines, heat exchangers, raw materials, active dry yeast, poorly stored backset (recycled thin stillage), or yeast slurry used as the inoculum (23). Microbial numbers can be significantly reduced by cleaning and sanitizing the equipment, by maintaining backset at a temperature over 70°C, by pasteurizing or chemically sterilizing the substrates, and by adding antibiotics, such as penicillin (22) or virginiamycin (11), to fermentors. In spite of these precautions, bacterial contamination still persists in many ethanol production plants. For example, in scotch malt whisky production plants where wort is not boiled in order to retain the activity of soluble enzymes of malt, bacterial contamination may compromise the quality of the distilled spirit and reduce the final yield of this high-value fermentation product (19). Isolates of lactic acid bacteria from distilleries are well-adapted to the conditions existing in such fermentations (4). In fact, enumeration of bacteria in many distilleries is often limited to the detection of lactic acid bacteria because aerobes and facultative anaerobes with little pH tolerance are not considered serious threats to product quality or production efficiency.

Yeasts and lactic acid bacteria are often encountered together in natural ecosystems and may be in competition for the same nutrients (1). When both microbes are grown together in a defined medium where yeast growth is restricted through provision of suboptimal concentrations of vitamins, a missing substance (nicotinic acid, adenine, guanine, aspartic acid, tryptophan, glycine, alanine, or lysine) essential for the growth of *Lactobacillus* spp. is synthesized in the medium by the yeast cells (5).

Uncertainties exist in the literature describing the effect of lactic acid bacteria on ethanol yield. Chin and Ingledew (6) reported that *Lactobacillus fermentum* inoculated at approximately 10^8 CFU/ml did not seriously affect ethanol productivity in the fermentation of diluted (14° Plato) wheat mash. Only moderate growth (two- to eightfold increases) of the bacteria occurred. Other workers, however, have reported that when bacterial numbers exceeded 10^8 CFU/ml at 30 h of fermentation, the spirit loss was approximately 5% (3, 7). According to Makanjuola et al. (19), reduced ethanol yields, lower yeast crops, reduced carbohydrate utilization, and an increase in acidity were all caused by the buildup of lactic acid. They found that a bacterial count of 4.5×10^8 CFU/ml at 30 h resulted in a 17% reduction in ethanol yield as a result of stuck fermentation. The concentrations of acetic acid (a minor end product of heterofermentative lactic acid bacteria and wild yeasts or a major end product of aerobic bacteria such as *Acetobacter* spp.) and lactic acid inhibitory to the growth of *S. cerevisiae* were 0.5 to 9 and 10 to 40 g/liter, respectively, and an 80% reduction in

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yeast cell mass occurred at concentrations of 7.5 and 38 g/liter, respectively (18). The toxic effect of lactic acid was enhanced further by an increase in osmotic pressure (8). A reduction in medium pH due to lactate production may also inhibit the saccharification process (12, 19). Despite a significant amount of research in the area, the effect of lactic acid bacteria on the rate and completion of yeast-catalyzed fermentations remains unclear (10). Any correlation between the extent of bacterial contamination and losses in alcohol yield has yet to be determined, although it is obvious that each molecule of sugar diverted to lactic acid production by the bacteria results in the loss of two molecules of ethanol that could have been produced by yeast cells (13). When bacterial contaminants are compared, the problem of the correlation of ethanol yield loss with the presence of lactic and/or acetic acid is complicated in part by the fact that homofermentative lactic acid bacteria metabolize glucose to lactic acid almost in stoichiometric quantities, whereas heterofermentative lactic acid bacteria produce lactic acid, CO_2, and ethanol and minor amounts of glycerol and acetic acid from the supplied glucose.

In this paper we describe the effect of five *Lactobacillus* isolates on alcoholic fermentation. The relationships between initial bacterial contamination of wheat mashes and losses in alcohol yield were studied. The effects of the bacteria and produced lactic acid on yeast growth and metabolism were also investigated.

MATERIALS AND METHODS

Bacteria used. Twelve species of lactobacilli were screened for growth rate in deMan-Rogosa-Sharpe (MRS) broth (Unipath, Nepean, Ontario, Canada) at 30°C and for alcohol tolerance. Five species that were capable of extensive growth in mash within 36 h and were tolerant to more than 10% (vol/vol) ethanol were selected for further study so that the information gained would be of use to the alcohol industry. Two organisms were obtained from Centro de Technologia Copersucar, Piracicaba, São Paulo, Brazil, and were tentatively identified to the species level and numbered biotype with API 50 CHL test kits (bioMérieux, Montreal, Quebec, Canada) as *Lactobacillus plantarum* biotype 1 and *Lactobacillus paracasei* subsp. *paracasei* biotype 2 (referred to as *L. paracasei* below). Two other strains, *Lactobacillus rhamnosus* ATCC 15280 and *L. fermentum* ATCC 14931, were obtained from the American Type Culture Collection. The fifth strain was an industrial isolate labelled Cargill #3 obtained from Cargill Corn Milling (Eddyville, Iowa). An API 50 CHI test kit for *Lactobacillus* identified this strain as *L. paracasei* subsp. *paracasei* biotype 2, but it differed in microscopic morphology and colony morphology from the *L. paracasei* strain obtained from Centro de Technologia Copersucar. Therefore, for the purposes of this study, we used the designation *Lactobacillus* #3.

Preparation of bacterial inocula. Lactobacilli were grown in 250-ml screw-cap sidearm Erlenmeyer flasks containing 50 ml of MRS broth. Then, 4-ml portions of late-log-phase cultures were transferred to 1-liter screw-cap flasks containing 200 ml of MRS broth. The headspace of each flask was flushed with filter-sterilized (0.22-μm-pore-size membrane filter) CO_2 gas, and the flasks were incubated in a model G25 Controlled Environmental shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 150 rpm and 30°C. Growth of the organisms was measured with a Klett Summerson colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a no. 66 red filter (420 to 660 nm), and the time for growth to the early stationary phase was determined. A relationship between Klett units and the number of CFU per milliliter in mid-log-phase cultures was established for each strain.

Bacterial cells (1,000 ml) were aseptically harvested by centrifugation at 10,200 × *g* for 15 min at 4°C (Sorvall model RC 5C centrifuge; GSA rotor; Sorvall Instruments, Division of Du Pont, Newtown, Conn.). The pellet was washed twice with sterile 0.1% (wt/vol) peptone water (Difco Laboratories, Detroit, Mich.), and the cells were then resuspended in 50 ml of sterile 0.1% (wt/vol) peptone water and chilled in ice until they were dispensed. Appropriate quantities of 20×-concentrated cell suspension were added to 500-g quantities of mash in fermentors to give final viable bacterial cell numbers of $\sim 10^5$, $\sim 10^6$, $\sim 10^7$, $\sim 10^8$, and $\sim 10^9$ cells (CFU)/ml. Volumes of inoculant were always made equal with sterile 0.1% (wt/vol) peptone water to avoid dilution of nutrients. In a control mash fermentation, bacteria were not inoculated, but yeasts were added at $\sim 10^6$ viable cells/ml. Growth and metabolism of bacteria in the absence of yeast cells were studied by inoculating mash with $\sim 10^7$ CFU of bacteria per ml.

Preparation of yeast inoculum. Eleven grams of *S. cerevisiae* active dry yeast (Allyeast superstart; Alltech, Inc., Nicholasville, Ky.) was dispersed into 99 ml of prewarmed (38°C), sterile 0.1% (wt/vol) peptone water and incubated at 38°C for 20 min with periodic shaking. Aliquots (0.25 ml) of this suspension were added to each fermentor to obtain $\sim 10^6$ viable yeast cells/ml.

Mashing of wheat and fermentation. Commercial red spring wheat bought from a local supplier was ground at setting no. 5 on a model S 500 disk mill (Glen Mills, Inc., Clifton, N.J.). For mashing, 19 liters of distilled water containing 1 mM $CaCl_2 \cdot 2H_2O$ was warmed to 60°C in a steam kettle. Seven kilograms of ground wheat was slowly added, and this was followed by 35 ml of high-temperature α-amylase (Alltech, Inc.). After 5 min, the temperature was raised to 90 to 95°C by using steam and held at this level for 45 min with stirring to gelatinize starch. The preparation was then cooled to 80°C by passing cold water through the jacket of the kettle, and a second 35-ml dose of high-temperature α-amylase was added. The mash was held for 30 min at this temperature to complete liquefaction of the gelatinized starch. The mash was strained under aseptic conditions through a sterile stainless steel food grade sieve (pore diameter, 1.5 mm), distributed into sterile bottles, and frozen at −40°C. Three days prior to fermentation, the mash was thawed, and 500-g quantities were aseptically transferred to sterile, jacketed, 1-liter Celstir bioreactors (Wheaton Instruments, Millville, N.J.). Diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.) was then added at a concentration of 0.01% (wt/vol) to sterilize the mash. The bioreactors were cooled immediately to 4°C by connecting them to a refrigerated water bath circulator and then stored at this temperature for 48 h.

The bioreactors were connected to a water bath circulator maintained at 30°C and stirred magnetically (IKA-Labortechnik, Staufen, Germany). One milliliter of filter-sterilized urea was added to each of the bioreactors to give a final concentration of 8 mM. Saccharification of dextrins to glucose was carried out by adding 0.8 ml of glucoamylase (Allcoholase II; Alltech, Inc.) per bioreactor 30 min before inoculation with yeast. Just prior to yeast inoculation, fermentors were contaminated (inoculated) with bacteria at the levels mentioned above. The temperature was maintained at 30°C throughout the fermentation. Samples were withdrawn for analysis from each fermentor every 6 h for the first 24 h and then at 36, 48, and 72 h.

Assay methods. (i) Viable counts of bacteria and yeast cells. Viable cell counts were monitored by the membrane filtration technique (14). For enumeration of yeast cells, the membranes were incubated aerobically at 30°C on the surfaces of YPD plates (10 g of yeast extract per liter, 10 g of peptone per liter, 20 g of dextrose per liter, 15 g of agar per liter) supplemented with 0.005% (wt/vol) gentamicin and 0.01% (wt/vol) oxytetracycline (Sigma Chemical Co.). The plating was done in triplicate for each dilution used.

Viable counts of bacteria on membrane-filtered samples were obtained by placing the filters on plates of MRS agar containing 0.001% cycloheximide (Sigma Chemical Co.) and incubating them in a CO_2 incubator (National Appliance Co., Portland, Oreg.) at 30°C after two cycles of evacuating and refilling with commercial-grade CO_2. The results were expressed as CFU per milliliter.

(ii) Determination of dissolved solids. Portions of samples were centrifuged at 10,300 × *g* for 30 min, and the supernatants were collected and stored in a freezer (−20°C) until they were analyzed. Total dissolved solids in these supernatants were determined by measuring the specific gravity at 20°C with a model DMA 45 density meter (Anton Parr KG, Graz, Austria). The readings were converted to grams of dissolved solids per 100 ml.

(iii) HPLC analysis. Ethanol and lactic acid were determined by high-performance liquid chromatography (HPLC) analysis. A 5-μl aliquot from a suitably diluted fermentation sample was analyzed by using a FAM-PAK column which analyzes sugars, alcohols, and organic acids (Waters Chromatographic Division, Milford, Mass.) maintained at 65°C. Orthophosphoric acid (1.5 mM) was used as the mobile phase at a flow rate of 1 ml/min. The components were detected with a differential refractometer (model 410; Waters Chromatographic Division). Methanol was used as the internal standard. The data were processed by using the Maxima 810 computer program (Waters Chromatographic Division).

RESULTS AND DISCUSSION

Fermentation rates. The disappearance of dissolved solids in the fermentation supernatant is a measure of glucose conversion to alcohol. Dissolved solids contribute to specific gravity, and the overall specific gravity decreases as sugar is converted to ethanol (density, 0.789) and CO_2 gas. Only small differences in fermentation rates were observed between the treatments containing *L. plantarum* and the controls with no bacterial inoculation (Fig. 1). Although a slight change in the rate of carbohydrate utilization was observed when the initial bacterial numbers were high, all of the fermentations completed (to constant specific gravity). This suggests that coflocculation (19) as a reason for incomplete utilization of carbohydrates and loss of ethanol yield due to high levels of bacterial contamination was not a factor here. When coflocculation exists, yeasts are unable to utilize all of the fermentable carbohydrates in the mash, resulting in stuck fermentation with residual unfer-

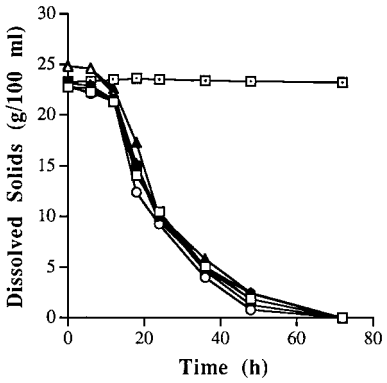

FIG. 1. Concentrations of dissolved solids over time in wheat mash fermentations at 30°C inoculated with various concentrations of *L. plantarum*. Most mashes were inoculated with yeast cells at approximately 10^6 CFU/ml. Symbols: □, control (no bacterial inoculation); ■, ~10^5 CFU of bacteria/ml; ○, ~10^6 CFU of bacteria/ml; ●, ~10^7 CFU of bacteria/ml; △, ~10^8 CFU of bacteria/ml; ▲, ~10^9 CFU of bacteria/ml; ⊡, ~10^7 CFU of bacteria/ml (no yeast inoculation).

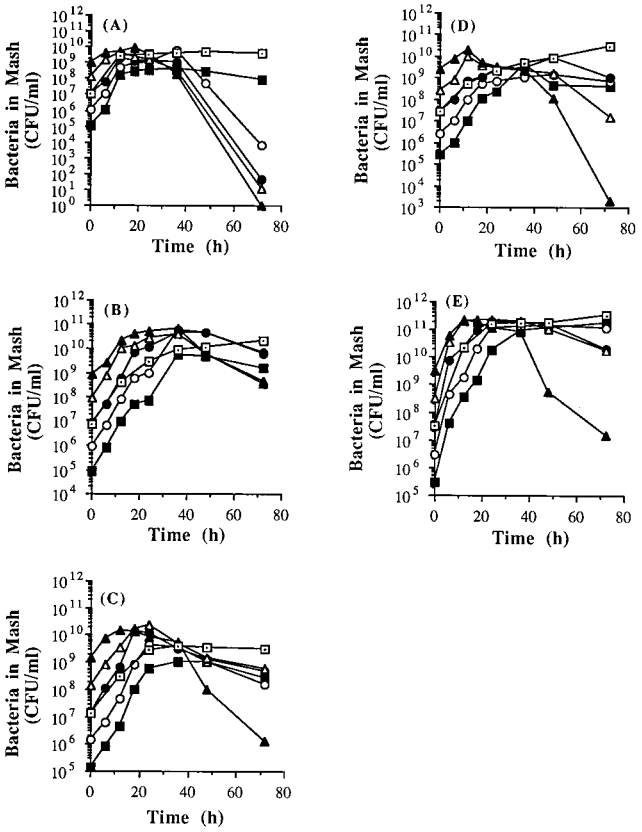

FIG. 2. Growth of *L. plantarum* (A), *L. paracasei* (B), *Lactobacillus* #3 (C), *L. rhamnosus* (D), and *L. fermentum* (E) inoculated at various levels into wheat mash fermented at 30°C. The mashes contained yeast cells at approximately 10^6 CFU/ml. Bacteria were inoculated at ~10^5 CFU/ml (■), ~10^6 CFU/ml (○), ~10^7 CFU/ml (●), ~10^8 CFU/ml (△), ~10^9 CFU/ml (▲), and ~10^7 CFU/ml (no yeast inoculation) (⊡).

mented sugar and a concomitant loss (up to 17%) of ethanol (19). Less than 0.1% fermentable sugars remained at the end of our fermentations, ruling out coflocculation.

When mash was inoculated with *L. plantarum* alone at approximately 10^7 CFU/ml, the bacteria did not consume more than 1 g per 100 ml of mash dissolved solids (fermentable carbohydrates) for growth and metabolism (Fig. 1). Similar trends were observed in the experiments done with *L. paracasei*, *Lactobacillus* #3, *L. rhamnosus*, and *L. fermentum* (data not shown). This implies that most glucose remained to be converted to ethanol by yeast cells, as suggested by Chin and Ingledew (6). However, when two organisms grow together in the same medium, there is always a competition between them for certain nutrients. The lactic acid bacteria anaerobically metabolize glucose to lactic acid to derive energy for growth and cell maintenance, and it is the production of lactic acid which leads to a reduction in ethanol yield (since every gram of lactic acid formed is at the expense of 0.51 g of ethanol).

Growth of lactobacilli in yeast-catalyzed fermentations. The growth of the five different lactobacilli inoculated at various levels into fermenting wheat mash is shown in Fig. 2. When these bacteria were grown in the absence of yeast cells, they remained viable, in contrast to the treatments with yeast cells, where they died off toward the end of fermentation. Moreover, the death rate of the bacteria increased with increases in the final concentration of lactic acid when lactic acid was present with ethanol. This suggests that ethanol acts synergistically with lactic acid to kill these bacteria and that the toxicity of ethanol is enhanced by the decrease in pH caused by the lactic acid in the medium. Based on the results obtained (Fig. 2), the homofermentative organisms *L. paracasei* and *Lactobacillus* #3 (since they did not die off as fast as the other organisms) appear to be more tolerant to ethanol than *L. rhamnosus* and *L. plantarum*. The former two organisms may be well-adapted to the prevailing conditions, as they are recent industrial isolates (4). In case of *L. fermentum*, a heterofermentative organism, the amount of lactic acid produced was only 0.5% (wt/vol), compared to *Lactobacillus* #3, which produced 1.59% (wt/vol) (Fig. 3). As the effect of ethanol is accentuated by the low pH of the medium due to lactic acid, *Lactobacillus* #3 died faster than *L. fermentum*, while *L. paracasei* was more tolerant to ethanol even at higher lactic acid concentrations in the medium.

When the bacteria were grown alone in the absence of yeast cells, their growth rates were initially slightly lower than those observed when they were grown in the presence of yeast cells. This might be attributed to the fact that these bacteria benefit from undetermined growth factors excreted by the yeast cells during growth (15, 17, 24). In contaminated yeast fermentations, the final lactic acid concentration measured at 72 h is proportional to the number of new bacterial cells produced. Once the maximum population is achieved, it is followed by the death of a proportion of the cells, while the survivors still metabolize glucose for cell maintenance. Therefore, when high numbers of cells are produced, higher rates of lactic acid production occur. Under anaerobic conditions, the bacteria derive energy by fermenting carbohydrates to lactic acid (homofermentative strains) or to a mixture of end products, such as ethanol, CO_2, and lactic acid, and minor end products, like acetic acid (heterofermentative strains).

Effect of lactobacilli on growth and metabolism of yeast cells. As the inoculum size of *L. plantarum* was increased, there were corresponding decreases in yeast growth rates. The growth rate of yeast cells in the fermentations with the smallest bacterial inoculum (10^5 CFU/ml) was 0.42 h^{-1}, while the growth rate with the largest bacterial inoculum (10^9 CFU/ml) was 0.36 h^{-1}. Decreases in the maximum yeast growth (Fig. 4), in the final ethanol concentration (Table 1), and in the final pH of the medium also were seen. This is in agreement with the results obtained by Makanjuola et al. (19). During fermenta-

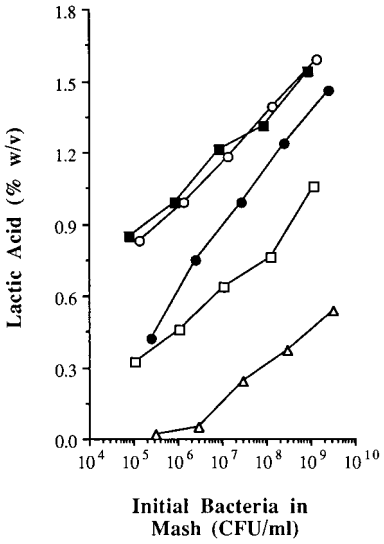

FIG. 3. Effect of the initial numbers of lactobacilli on the final lactic acid concentration. The mashes contained yeast cells at approximately 10^6 CFU/ml. Symbols: □, *L. plantarum*; ■, *L. paracasei*; ○, *Lactobacillus* #3; ●, *L. rhamnosus*; △, *L. fermentum.*

tion, the concentrations of lactic acid achieved increased as the levels of bacteria inoculated increased (Fig. 3). This was also observed by other authors (3, 19). In all experiments, the total yeast cell numbers reached a maximum at 24 h of fermentation and then started to decrease (Fig. 4). Only data pertaining to the *L. plantarum* coinoculation experiment are shown. The trend was similar with the other four strains (data not shown). The effects of each *Lactobacillus* sp. at the five different inoculation levels on the maximum amount of ethanol produced by yeast cells were studied in separate sets of experiments (Table 1). Single mashes were used for each experiment because between experiments there were minor changes in the dissolved solid contents of the mashes that would have led to variations in the final ethanol concentrations produced by yeast cells in the absence of bacteria. Yeast-mediated fermentations, as described above, are over within 48 h. Therefore, even when bacteria are present in high numbers, they must increase in

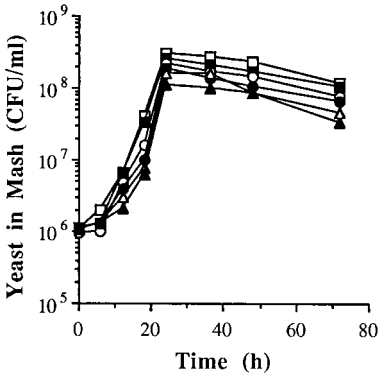

FIG. 4. Growth of yeast cells in fermenting wheat mash at 30°C coinoculated with *L. plantarum* at various levels. The mashes contained yeast cells at approximately 10^6 CFU/ml. Symbols: □, control (no bacterial inoculation); ■, ~10^5 CFU of bacteria/ml; ○, ~10^6 CFU of bacteria/ml; ●, ~10^7 CFU of bacteria/ml; △, ~10^8 CFU of bacteria/ml; ▲, ~10^9 CFU of bacteria/ml; □, ~10^7 CFU of bacteria/ml (no yeast inoculation).

TABLE 1. Concentration of ethanol produced after fermentation of normal-gravity (22 to 24° Plato) wheat mash at 30°C for 72 h by yeast cells coinoculated with lactobacilli at various levels[a]

Approx no. of bacteria inoculated (CFU/ml)	Maximum concn of ethanol produced (%, vol/vol)[b]				
	L. plantarum	*L. paracasei*	*Lactobacillus* #3	*L. rhamnosus*	*L. fermentum*
None (control)	12.71	12.46	12.24	12.71	13.14
10^5	12.55	12.20	11.99	12.50	13.04
10^6	12.40	11.99	11.77	12.32	12.90
10^7	12.31	11.80	11.60	12.20	12.82
10^8	12.19	11.65	11.44	12.07	12.75
10^9	11.99	11.51	11.30	11.86	12.63

[a] Mashes were inoculated with approximately 10^6 CFU of yeast cells/ml.
[b] All assays were done in duplicate with HPLC analysis. The variations in ethanol concentrations (in duplicate assays) were in all cases less than 0.04% (vol/vol).

biomass quickly in order to create enough metabolic potential to compete with yeast cells for sugar and create ethanol yield-reducing levels of lactic acid prior to termination of fermentation.

The increases in lactic acid with the five bacterial strains correlated with the decreases in the final ethanol concentrations (correlation coefficients, 0.92 to 0.99) and the viable yeast numbers. The production of the metabolic end product of these bacteria, lactic acid, inhibits yeast growth and metabolism (18) and was the major cause of the decrease in ethanol yield in this study. In the case of *L. fermentum*, a heterofermentative organism, the lactic acid concentrations finally achieved were not as high as those achieved with the homofermentative strains used in this study (Fig. 3), yet the percent reduction in ethanol production by yeast cells was comparable to that of the control (about 2%) when the preparations were inoculated at approximately 10^6 CFU/ml with the homofermentative organisms (*L. plantarum* and *L. rhamnosus*). This may be partly due to the production of 0.03 to 0.05% (wt/vol) acetic acid by *L. fermentum* toward the end of fermentation (the actual amount depended on the inoculation level). Acetic acid is more toxic than lactic acid since it has a higher pK_a than lactic acid. It is the undissociated form of the organic acid that is responsible for antimicrobial activity (2), and therefore a higher concentration of the undissociated form of acetic acid exists at the pH values of mash fermentation. The two acids together have been shown to have a synergistic and negative effect on yeast growth and metabolism (20). It has also been found that ethanol accentuates the effect of acetic acid in the inhibition of fermentation by yeast cells (21). Lactic acid and acetic acid along with ethanol should have acted synergistically on yeast growth and metabolism, resulting in a decrease in ethanol yield. It has been reported that acetic acid, in its undissociated form, causes an increased expenditure of energy (ATP) for cell maintenance (18). However, data for the action of acetic acid and lactic acid on yeasts show growth inhibition different from that predicted on the basis of dissociation constants, indicating that these acids may not act in the same manner on yeast cells (18, 20).

Lactobacilli are extremely fastidious. They require a variety of growth factors, like nucleotides, amino acids, and vitamins (15). Biotin and vitamin B_{12} are required by a few strains (15, 16). Biotin is also an essential growth factor for *S. cerevisiae* (16). Therefore, lactobacilli, when they are present in high numbers, can quickly scavenge from the medium large amounts of the essential growth factors required by the yeast cells. Since the growth rates of the selected lactobacilli are

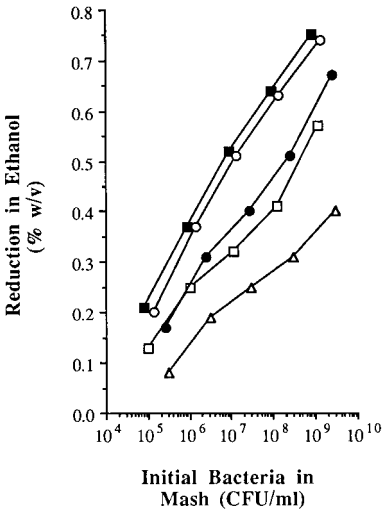

FIG. 5. Effect of the initial numbers of lactobacilli on the reduction in final ethanol concentration compared to the control with no bacterial inoculation. The final ethanol concentrations (Table 1) were approximately 10% (wt/vol) (12.7%, vol/vol), so the losses in produced ethanol ranged from 0 to 7.5%. The mashes contained yeast cells at approximately 10^6 CFU/ml. Symbols: □, *L. plantarum*; ■, *L. paracasei*; ○, *Lactobacillus* #3; ●, *L. rhamnosus*; △, *L. fermentum.*

higher than the growth rates of *S. cerevisiae* and lactic acid bacteria found in breweries and food products, removal of essential growth factors could result in reductions in the yeast growth rate and catalytic activity. This would reduce the final ethanol yield.

Results obtained in this study show that initial bacterial contamination of mash with approximately 10^6 CFU/ml led to as much as a 2% reduction in ethanol yield compared to the control with no bacteria (Table 1). Higher levels of bacteria (10^9 CFU/ml) led to greater than 7% losses in produced ethanol. In fact, both final lactic acid concentrations and decreases in ethanol yields at the end of fermentation were directly correlated with initial numbers of viable bacteria in the mash (Fig. 3 and 5). This is the first report which shows that there is a linear relationship among these parameters. Likewise, there is a relationship among the metabolic pathways (heterofermentation versus homofermentation), the growth rates, and the lactic acid produced by these bacteria. In fact, the growth of the heterofermentative strain as indirectly measured by optical density in MRS broth was less than the growth of homofermentative strains, which, via glycolysis, produced twice as much ATP per molecule of glucose fermented. This agrees with the theory concerning the relationship of ATP to biomass in anaerobically grown bacteria, and twice as much lactic acid is produced by homofermentative lactic acid bacteria as by heterofermentative strains (9).

As little as a 1% decrease in ethanol yield is highly significant to distillers of fuel alcohol since their profit margins are very narrow (19). In large plants with outputs of 400 million to 1,100 million liters of ethanol per year, such a decrease would reduce income by $1 million to 3 million annually. Significant decreases (more than 2%) in ethanol concentrations were observed with *L. paracasei* and *Lactobacillus* #3, even when they were inoculated at a low level, 10^5 CFU/ml. This may be because they did not die off toward the end of fermentation as the other strains used in this study did (Fig. 2). These observations are in contrast to those reported by Dolan (7) and Barbour and Priest (3) from industrial-scale studies.

The reduction in the pH of the medium due to lactic acid production did not affect the saccharification process. When the activity of the glucoamylase used was assayed at various pH levels, it was found that the enzyme retained about 91% of its original activity at pH 4.0 and 70% of its original activity at pH 3.0 (data not shown). It has been reported that there is a significant loss in glucoamylase activity when the pH falls below 3.5 (6), but the lowest pH observed in the present study was only 3.9.

As mentioned by Makanjuola et al. (19), studies on the direct effects of contamination are not easily carried out. The differences between and within batches of raw materials produce differences in chemical composition and measured fermentation parameters. Raw materials themselves may harbor contaminating bacteria which compete with yeasts for growth-promoting nutrients. In this study, the effects of five contaminating lactobacilli were studied under conditions where all factors other than initial numbers of bacteria present in the mash prior to yeast inoculation were controlled.

The results indicate that apart from the diversion of small amounts (less than 1%, wt/vol) of fermentable sugar for growth of the bacteria, the production of lactic acid and a suspected competition by the bacteria for nutrients that promote yeast growth are the important reasons for the reductions in yeast growth and metabolism and, ultimately, the final ethanol yield. Little research has been done to determine the exact mechanism of action of lactic acid on yeast growth and metabolism. Such work is in progress.

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